FACSCalibur Set-up Using CellQuest Pro Software

1.   Turn on the FACSFlow Supply System located under the bench (green switch on right side).

Indicator Lights   Power Switch
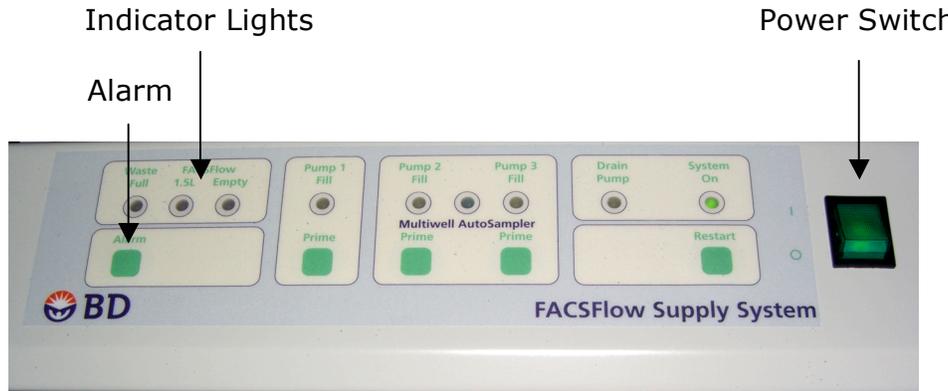
Alarm

a) Check the indicator lights to see if the waste is full or the sheath is empty. If the fluidics need to be changed, please seek guidance from a qualified technician if you are not able.

b) During acquisition, an alarm may sound if the fluids need to be changed. The alarm may be silenced by depressing the alarm button.

2.   Turn the instrument on (green button on right/rear side panel).

3.   Turn on the computer. The cytometer MUST be turned on before the computer.

4.   Press **PRIME** to drain and fill the flow cell.

5.   Open Acquisition template.

a) If you have saved an acquisition template under your Client Folder, open it by double-clicking (look for acquisition icon). This will launch CellQuest Pro and open the template.

b) If you are using a generic template, open the folder labeled **Acquisition** under **Data** and double-click on the appropriate template (do not overwrite these templates. Select "Don't Save" when closing a template unless you are saving it to your folder).

c) Alternately, if CellQuest has already been launched, you may open an acquisition template by selecting the **File** drop down menu and selecting **Open Document**.
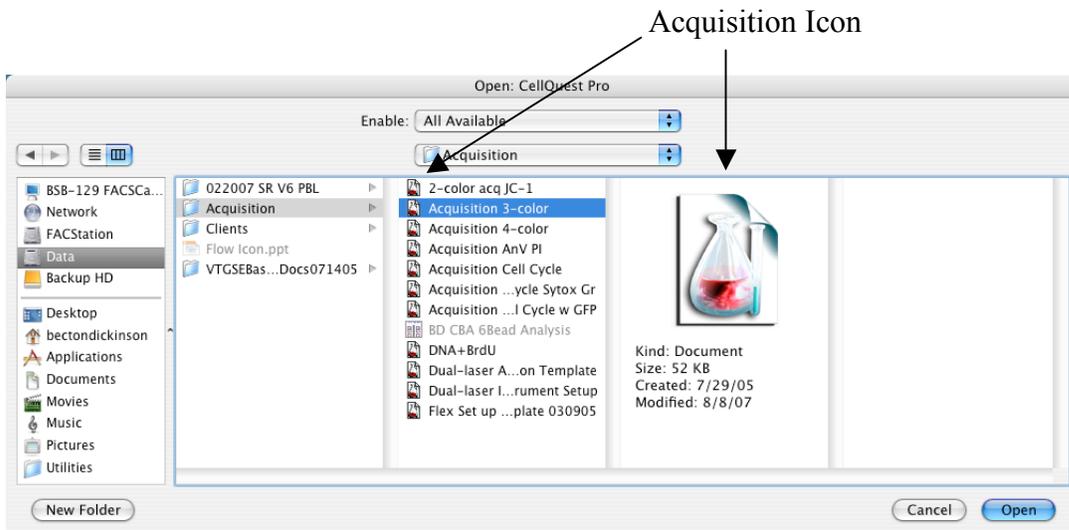
Acquisition Icon

6. From the **Acquire drop down menu** select:

    a) **Connect to Cytometer**. The **Acquisition Control** toolbar will automatically appear. If you do not see the Acquisition tool bar, go to the **Windows** drop down menu and select **Show Acquisition Tool Bar**.

    Notice that the **Setup** box is checked. No data is saved in the setup mode. Once check is removed the files will be automatically saved when the desired count is achieved.

    b) **Counters**. Move it to a desirable position on the screen. Expand with counter window by selecting the green dot and change the second Accept to Collect. This will allow you to see what is actually being collected in region/gate R1/G1.
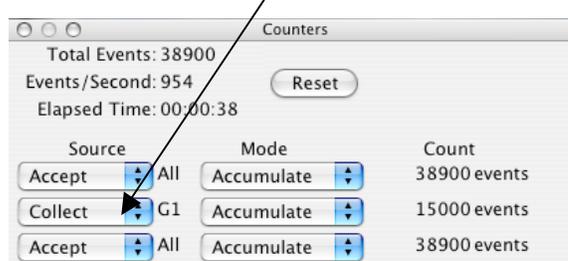
c) **Parameter Description**. Browser window will open. Move it to the upper right of the screen. Notice that it also has an acquisition control. You can use either one to acquire data. In this window you will need to change the **Directory**, **File**, **Sample ID**, and **Parameter Settings**.
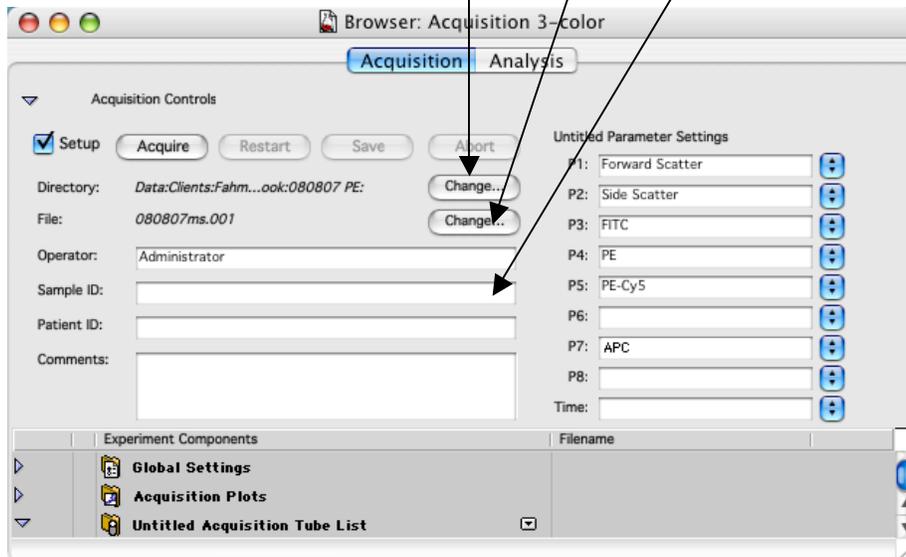
i) **Change Directory**. Select **Change** and go to your folder (Data>Clients>Your Name>New Folder). Create a **New Folder** then select **Choose** (Please do not make changes to other clients folders).
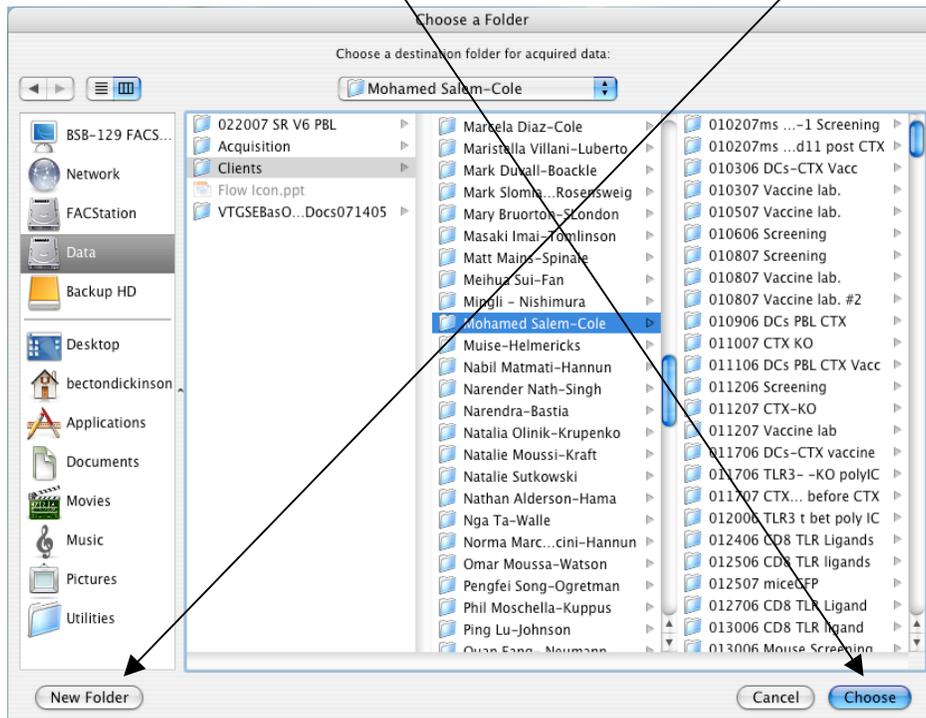
ii)   **Change File**. Select **Change** and rename the **Custom Prefix**.
I typically use the date and the user's initials (i.e. 080807ms).
You may change it to whatever suits you. Reset the **File Count**
to 1 (Remember, if you need to reacquire data that has already
been saved, you will need to change the file count to overwrite
the old data). Select OK.
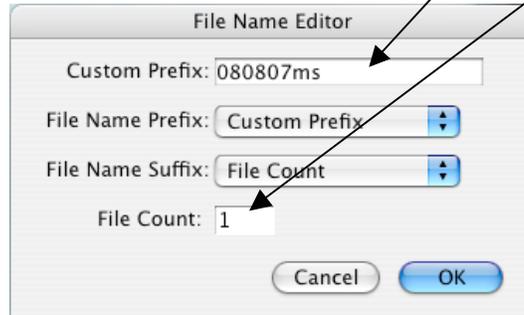
iii)   **Sample ID.** Change to describe your sample.

iv)   **Parameter Setting.** Change to describe the antibodies, dyes,
and fluorochromes used in your experiment. You may type or
use the pull down menu.

d) **Acquisition and Storage.** This is found under the **Acquire** pull
down menu. In this window you can change the number of events
to be counted and the gate. 10,000 to 20,000 cells is standard
depending on the experiment. A time limit option is also available
for slow running samples (i.e. 10,000 cells or 120 seconds –
whichever comes first).
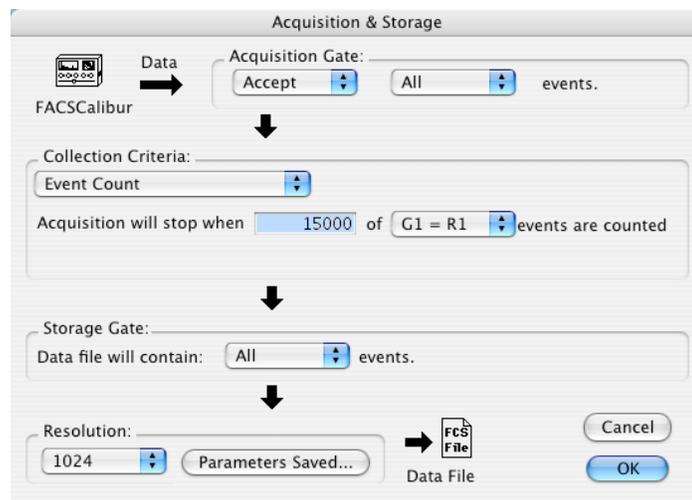
7. From the **Cytometer** drop down menu select **Detectors/Amps**,
Compensation, **Threshold**, and **Status** (Alternately, you may choose 1,
2, 3, 4). I typically move these to the lower left side of the screen.
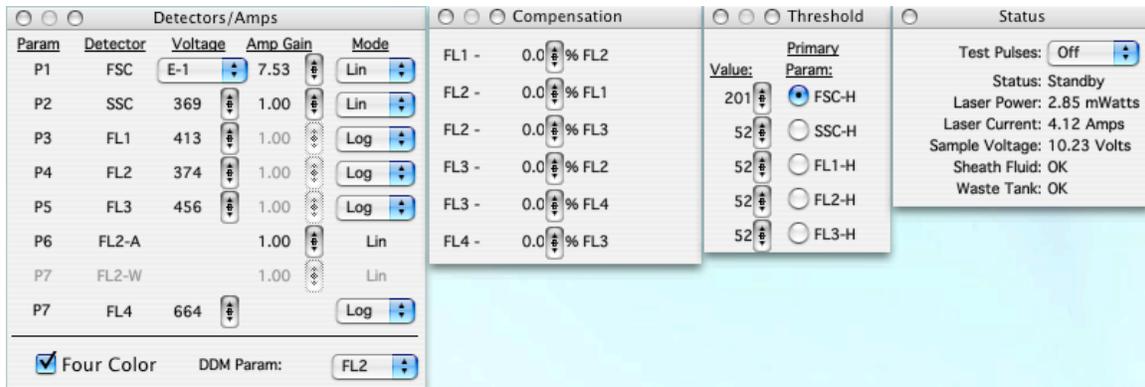
a) **Detectors/Amps.** This window is used to adjust the **Voltage** and **Amp Gain** of the detectors.

   i) **Forward Scatter** (FSC/P1) - This parameter is detected using a photodiode and is a measure of cell size. FSC can be adjusted using the Voltage and Amp Gain. Logarithmic increments of E01, E02, and E03 increase the signal of the small events; E-1 reduces the signal of large events. For most applications the voltage is set to E-1 or E1.

   ii) **Side Scatter** (SSC/P2) – This parameter is detected using a photomultiplier tube (PMT) and is a measure of cell complexity. This signal is amplified by applying a voltage to the PMT. As the voltage is increased, the detector sensitivity increases.

   iii) **FL1 (P3), FL2 (P4), FL3 (P5), and FL4 (P7) –** These parameters are also detected by PMTs and are a measure of fluorescent intensity. As the SSC, these parameters are amplified by applying a voltage to the PMTs.

   iv) **Four Color –** Checking this box simply turns on the 635nm laser. Turn this laser on whenever you are using 635nm excitable fluorochromes such as APC or Alexa 647.

   v) **Mode – Log** amplification is often used to analyze samples with a large dynamic range of fluorescence signals. The log scale has four decades of range. For most applications, such as phenotyping, the fluorescence parameters are set to log.
Linear amplification is usually used for light scatter parameters. It is also used for DNA cell cycle analysis. The linear amplifier gain can be adjusted from 1.00-9.99.

b) **Threshold.** In this window, anything below the selected channel number will not be processed. You can use two threshold parameters. When running immunophenotyping, the threshold is set on FSC to eliminate events such as

debris. When running cell cycle analysis, the threshold can be set on FL2. Do not use a threshold at or near 0. This will introduce a lot of unwanted electronic noise. The default setting is 52.

c) **Compensation.** This allows you to adjust for spectral overlap when samples are stained with two or more fluorochromes. Fluorochromes emit light over a range of wavelengths. As a result, a signal from one fluorochrome might appear in a detector used for another fluorochrome. For example, FITC appears primarily in the FL1 detector, but some of the fluorescence overlaps into the FL2 detector. PE appears primarily in the FL2 detector, but some of the fluorescence overlaps into FL1 and FL3 detectors. Compensation allow you to eliminate the spectral overlap electronically.

8. Instrument Settings.

a) Importing instrument settings. If you have saved settings open them by opening the **Cytometer** drop down menu and select **Instrument Settings.** Select **Open** and navigate to your settings file then select **Set** and **Done.**
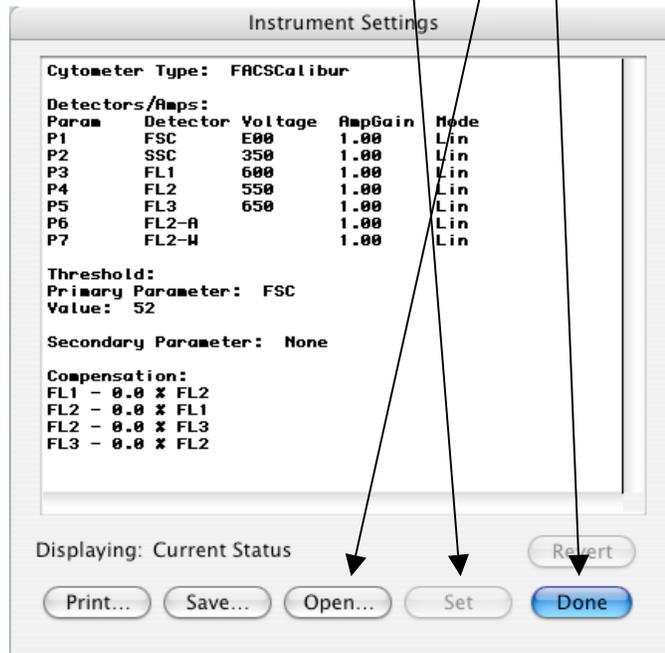
Tip: Your old data files (fcs files) contain the instrument setting used to generate them. You may choose an fcs file that has similar settings for the experiment that you are running for that day. Again, select Cytometer>Instrument Settings>Open>Select FCS File>Set>Done.

b) <mark>If you do <u>not</u> have saved settings</mark>.

i) Press the Run button on the face of the machine and put your negative control on the SIP. Be sure to engage the tube arm immediately or it will aspirate your sample.

ii) While your control is running <mark>adjust the FSC amp gain and voltage (E00 or E-1) and SSC amp gain</mark> so that the population of interest is clearly visible towards the center of FSC vs SSC dot plot. Make sure FSC and SSC are in the linear (lin) mode.

| SCATTER ON SCALE | SSC TOO LOW | FSC TOO LOW |
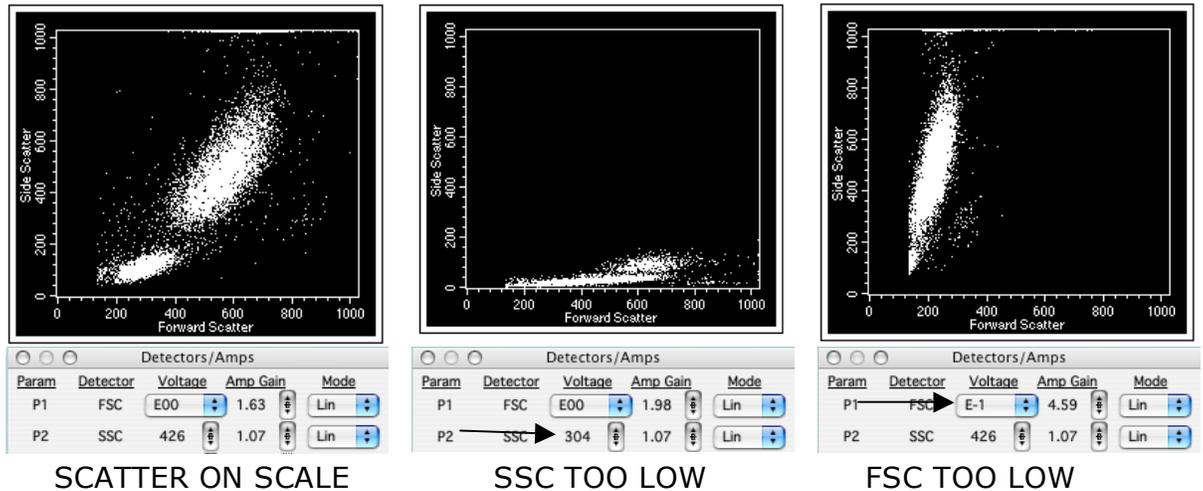
iii) <mark>Adjust the threshold</mark> on FSC so that most of the debris is eliminated.

iv) You may <mark>draw a gate</mark> around the population of interest to remove unwanted debris and aggregates. Do so by selecting a circle or polygon from the **Tool Palette**.
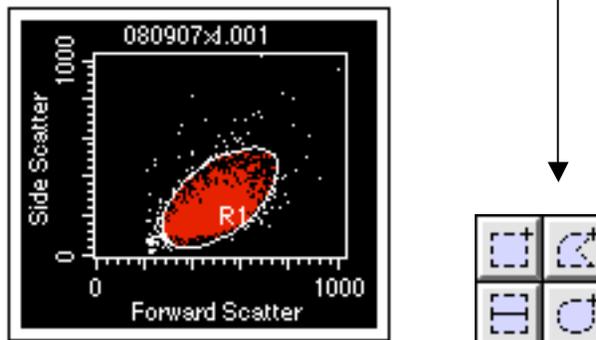
v) You must remember to <mark>activate the gate</mark> in subsequent plots. You may do so under Gates in the Inspector window (Window>Show Inspector>Gate>Select Region).

vi) <mark>Adjust the voltage on all fluorescence parameters</mark> (FL1, FL2, FL3, FL4) so that the autofluorescence falls in the middle of the first log.
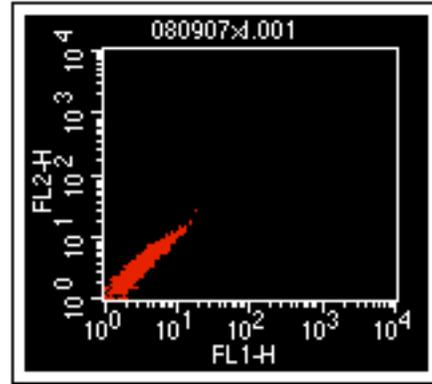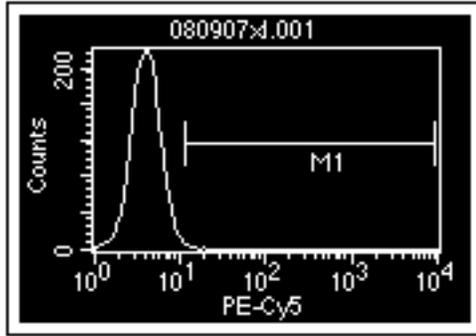
vii) <mark>Adjust Compensation</mark>.

    1) Put on your first compensation control (i.e. FITC). You may notice that the FITC signal fluoresces mostly in the FL1 channel, but bleeds over into the FL2 channel. This needs to be compensated.

    2) Go to the compensation window that you opened previously and adjust the equation F2 - ___%FL1

    3) View FL1 vs FL2 dot plot. As you increase the % compensation watch as the FITC+ population is subtracted from the FL2 channel and placed solely in the single positive quadrant.

    4) Be careful not to over compensate. The median of the FITC+ population should equal that of the unstained population.

PROPERLY COMPENSATED   UNDERCOMPENSATED   OVERCOMPENSATED
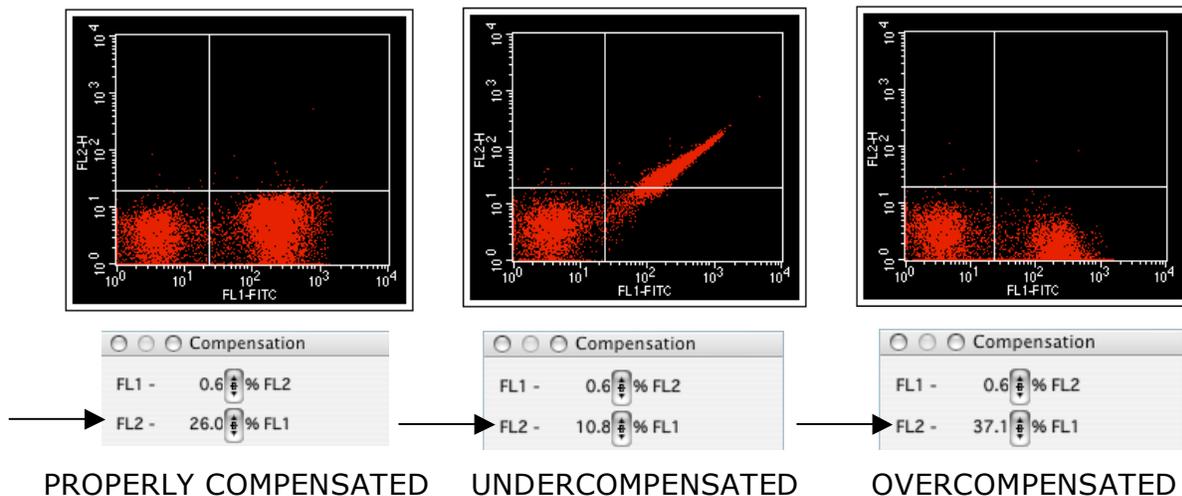
    5) You will need to repeat this compensation adjustment for each fluorochome combination. See the compensation guide below or ask for assistance for additional instructions.

    6) Once the compensation is complete do not change the voltages. Doing so will unbalance the compensations and the process will have to be repeated.

9.   Once you are sure that your settings are correct you may start acquisition. <mark>Uncheck the **Setup** button and click **Acquire** in the **Browser** window</mark>.

        When the computer beeps and the counters stop, the data file is automatically saved to your folder. Change the sample ID and acquire the next sample. To save the data file before it has reached the total event count click **Pause** then **Save** in the **Acquisition Control** or **Browser** window.

10.   <mark>Machine cleaning shut down procedure</mark>.

        a) Run 10% bleach or FACSClean on HI for 5 minutes.
        b) Run ddH$_2$O on HI for 5 minutes.
        c) Put the cytometer in Standby. The tube of ddH$_2$O should remain on the SIP.
        d) Shut down computer (shutting down the cytometer before the computer will cause the computer to hang up).
        e) Turn off the FACSCalibur.
        f) Turn off the FACSFlow Supply System.

Trouble Shooting Guide

I put my cells on the SIP, pressed RUN and Acquire, but I can't see any event on the screen.

1. Make sure the Run button is green and not orange. If it remains orange then there is a pressurization problem. Check to see if there is crack in the tube or if the pressure relief valve in the fluidics drawer is on vent. Replace the tube and/or flip the vent switch so that it is toward you. Also, the diameter of the sample tube opening may be too large. Please order BD Falcon 352054 tubes to ensure a proper fit.
2. You may have a clog in the SIP. Replace the sample tube with ddH$_2$O and press Prime. Repeat if this does not work the first time. If the machine keeps clogging then you will need to put your samples through a cell strainer.
3. Increase your FSC gain; your cells may be below threshold.
4. Try another sample; there may be no cells in your tube.
5. If none of these resolves your problem, seek guidance from the Flow Lab staff.

When I open up CellQuest Pro, I cannot "Connect to the Cytometer"

1. Make sure the cytometer is turned on.
2. Quit CellQuest and restart the computer.

I cannot find my data.

1. Check the Directory path under the Browser window if it is still open.
2. Do a search by directory and/or date.

Compensation Guide

Compensation for FL1 vs FL2
BP Filter - 530/30
Probes - FITC, GFP, Alexa488, CFSE, DCF

Under Compensated	Over Compensated	Properly Compensated

FL2 - ___% FL1
Increase Value

FL2 - ___% FL1
Decrease Value
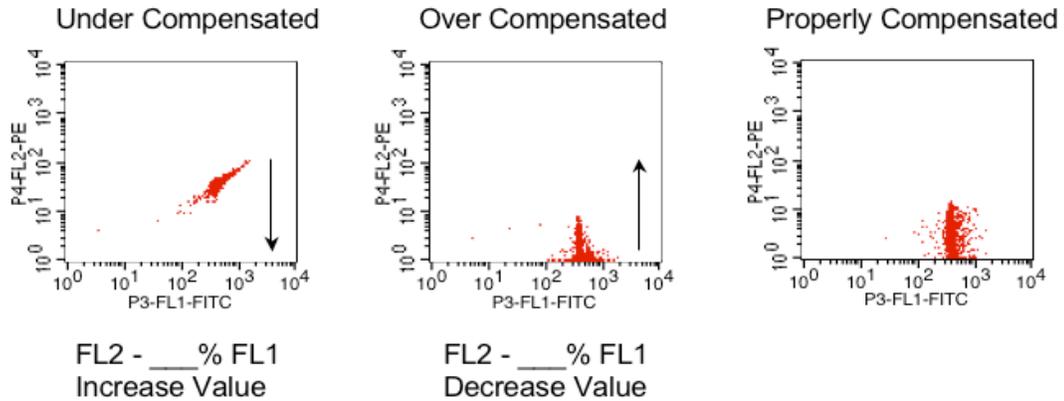
Compensation for FL1 vs FL2 & FL2 vs FL3
BP Filter - 585/42
FL2 Probes - PE, PI, PKH 26, DsRed

Under Compensated	Over Compensated	Properly Compensated

FL1 - ___% FL2
Increase Value

FL1 - ___% FL2
Decrease Value

FL3 - ___% FL2
Increase Value

FL3 - ___% FL2
Decrease Value
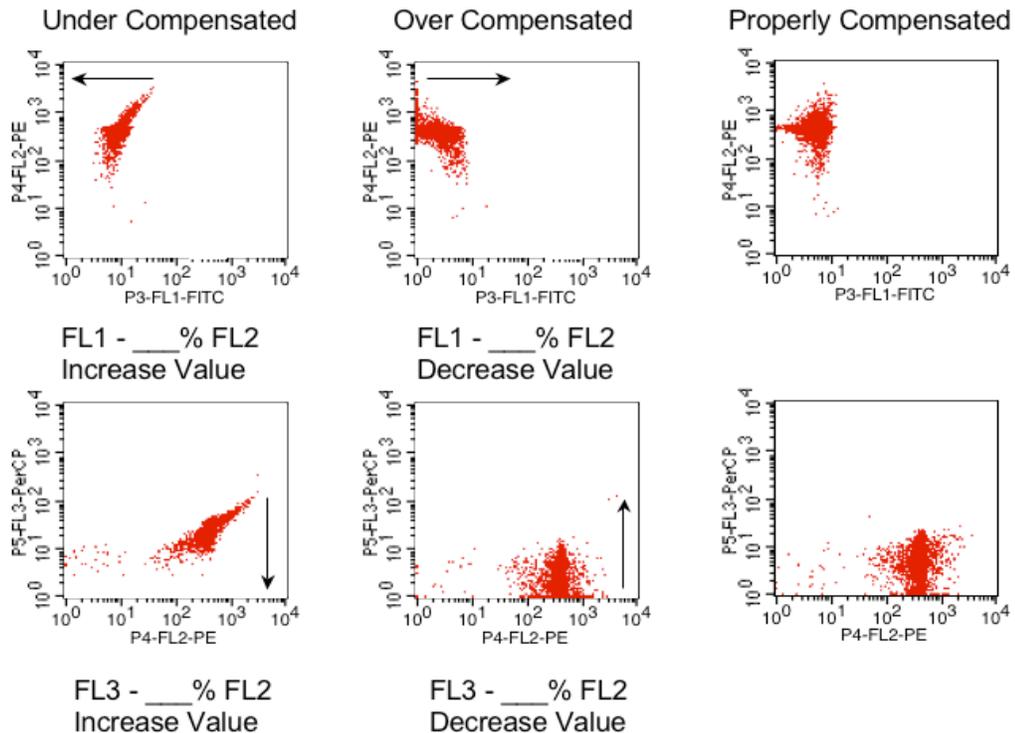
Compensation for FL2 vs FL3 & FL3 vs FL4

LP Filter - > 670

FL3 Probes - PerCP, PE-Cy5, PE-Cy7, 7-AAD

Under Compensated	Over Compensated	Properly Compensated

FL2 - ___% FL3
Increase Value

FL2 - ___% FL3
Decrease Value

FL4 - ___% FL3
Increase Value

FL4 - ___% FL3
Decrease Value
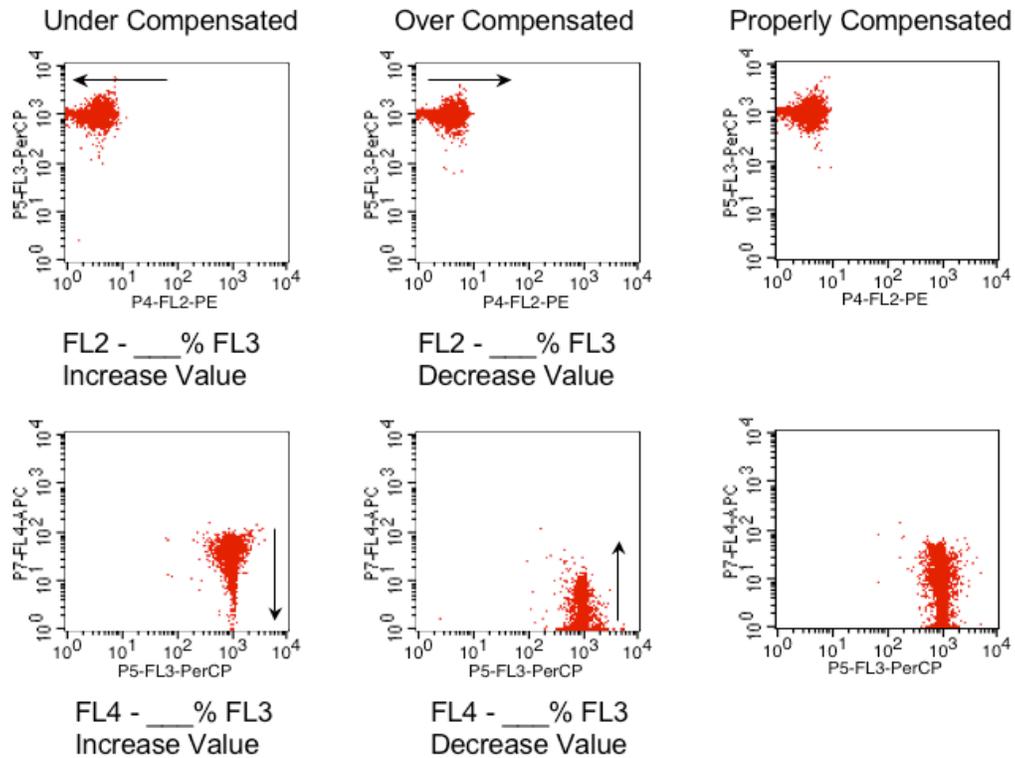
Compensation for FL3 vs FL4

BP Filter - 661/32

Probes - APC, Alexa647, Cy5

Under Compensated	Over Compensated	Properly Compensated

FL3 - ___% FL4
Increase Value

FL3 - ___% FL4
Decrease Value

FACSCalibur				
PMT Designation	"FL1"	"FL2"	"FL3"	"FL4"
Filter Specs	530/30 BP	585/42 BP	670 LP	661/16 BP

488 nm laser ──── **635 nm laser**

Fluorochromes	Fluoriscein (FITC)	Phycoerythrin (PE)	PerCP	APC
	GFP	Propidium Iodide (PI) →	PE-Cy5	Alexa 647
	Alexa 488	PKH 26	7-AAD	Cy5
	CFSE	DsRed	PerCP-Cy5.5	ToPro-3
	PKH 67	Vybrant Orange	PE-Cy7	
	Vybrant Green		DRAQ5	
	DCF			

FL1 - ____%FL2   FL3 – ____%FL2

PE Neighbors

Here is a handy diagram of fluorochromes and dyes that can be used on FACSCalibur. Neighboring fluorochromes need to compensated (i.e. PE(FL2) needs to be compensated from FITC(FL1) and PE-Cy5(FL3)). Note: Although PI is commonly used in FL2, PI is strongest in FL3 and should be avoided when using other FL3 fluorochromes.